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Specification and Drawings, as originally filed, with Application for Patent Serial No:
2,321,256, on October 16, 2000, by **PROCYON BIOPHARMA INC.**, for
"Pharmaceutical Preparations and Methods for Inhibiting Tumours".


L. Legimbal
Agent certifié / Certifying Officer

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Date

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**PHARMACEUTICAL PREPARATIONS AND METHODS
FOR INHIBITING TUMOURS**

FIELD OF THE INVENTION

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The present invention relates to pharmaceutical preparations for use as tumour suppressive agents for tumours arising from cancers such as prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial and ovarian cancers, and
10 benign prostate hyperplasia (BPH).

BACKGROUND OF THE INVENTION

The prostate gland, which is found exclusively in male
15 mammals, produces several components of semen and blood and several regulatory peptides. The prostate gland comprises stroma and epithelium cells, the latter group consisting of columnar secretory cells and basal nonsecretory cells. A proliferation of these basal cells as well as stroma cells gives
20 rise to benign prostatic hyperplasia (BPH), which is one common prostate disease. Another common prostate disease is prostatic adenocarcinoma (CaP), which is the most common of the fatal pathophysiological prostate cancers, and involves a malignant transformation of epithelial cells in the peripheral region of
25 the prostate gland. Prostatic adenocarcinoma and benign prostatic hyperplasia are two common prostate diseases which have a high rate of incidence in the aging human male population. Approximately one out of every four males above the age of 55 suffers from a prostate disease of some form or
30 another. Prostate cancer is the second most common cause of cancer related death in elderly men, with approximately 96,000 cases diagnosed and about 26,000 deaths reported annually in the United States.

35

Studies of the various substances synthesized and secreted by normal, benign and cancerous prostates carried out

in order to gain an understanding of the pathogenesis of the various prostate diseases reveal that certain of these substances may be used as immunohistochemical tumour markers in the diagnosis of prostate disease. The three predominant

5 proteins or peptides secreted by a normal prostate gland are:

(1) Prostatic Acid Phosphatase (PAP); (2) Prostate Specific Antigen (PSA); and, (3) Prostate Secretary Protein of 94 amino acids (PSP⁹⁴), which is also known as Prostatic Inhibin Peptide (PIP), Human Seminal Plasma Inhibin (HSPI), or β -

10 microseminoprotein (β -MSP), and which is hereinafter referred to as PSP⁹⁴.

PSP⁹⁴ is a simple non-glycosylated cysteine-rich protein, and constitutes one of three predominant proteins found
15 in human seminal fluid along with Prostate Specific Antigen (PSA) and Prostate Acid Phosphatase (PAP). PSP⁹⁴ has a molecular weight of 10.7 kDa, and the complete amino acid sequence of this protein has already been determined (SEQ ID NO:1). The cDNA and gene for PSP⁹⁴ have been cloned and characterized (Ulvback, et
20 al., Biochem. Biophys. Res. Comm., 164:1310, 1989; Green, et al., Biochem. Biophys. Res. Comm., 167:1184, 1990).

Immunochemical and in situ hybridization techniques have shown that PSP⁹⁴ is located predominantly in prostate epithelial cells. It is also present, however, in a variety of other secretory
25 epithelial cells (Weiber, et al., Am. J. Pathol., 137:593, 1990). PSP⁹⁴ has been shown to be expressed in prostate adenocarcinoma cell line, LNCap (Yang, et al., J. Urol., 160:2240, 1998). As well, an inhibitory effect of exogenous PSP⁹⁴ on tumour cell growth has been observed both in vivo and in
30 vitro (Garde, et al., Prostate, 22:225, 1993; Lokeshwar, et al., Cancer Res., 53:4855, 1993), suggesting that PSP⁹⁴ could be a negative regulator for prostate carcinoma growth via interaction with cognate receptors on tumor cells.

Native PSp⁹⁴ has been shown to have a therapeutic modality in treating hormone refractory PCa (and potentially other prostate indications).

Metabolic and immunohistochemical studies have shown that the prostate is a major source of PSp⁹⁴. PSp⁹⁴ is involved in the feedback control of, and acts to suppress secretion of, circulating follicle stimulating hormone (FSH) both in-vitro and in-vivo in adult male rats. PSp⁹⁴ acts both at the pituitary as well as at the prostate site since both are provided with receptor sites for PSp⁹⁴.

Both PSA and PAP have been studied as tumour markers in the detection of prostate disease, but since both exhibit elevated levels in prostates having benign prostatic hyperplasia (BPH), neither marker is specific and therefore they are of limited utility.

Recently, it has been shown that PSp⁹⁴ concentrations in serum of patients with BPH or CaP are significantly higher than normal. The highest serum concentration of PSp⁹⁴ observed in normal men is approximately 40 ng/ml, while in men with either BPH or CaP, serum concentrations of PSp⁹⁴ have been observed in the range from 300-400 ng/ml. Because there exists some overlap in the concentrations of PSp⁹⁴ in subjects having normal prostates and patients exhibiting either BPH or CaP, serum levels in and of themselves are of little value.

A major therapy in the treatment of prostate cancer is androgen-ablation. While most patients respond initially to this treatment, its effectiveness decreases over time, possibly because of the presence of a heterogeneous population of androgen-dependant and androgen-independent cells to the androgen treatment, while any androgen insensitive cells present would continue to proliferate unabated.

Other forms of cancer which are currently exacting a heavy toll are breast cancer in women and cancer of the gastrointestinal tract. Currently, the use of various cancer drugs such as mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin and daunomycin form part of the therapy for treating such cancers. One drawback to such a therapeutic treatment is the presence of adverse side effects due to the drugs in the concentration ranges required for effective treatment.

Accordingly, it would be advantageous to find a more effective means of arresting the growth of prostate, breast and gastrointestinal cancer cells and tumours which can be used effectively against both androgen sensitive and androgen insensitive cells.

In our previous work, described in United States Patent No. 5,428,011, we provided pharmaceutical preparations of native human seminal plasma PSP⁹⁴ for inhibiting in-vitro and in-vivo cancerous prostate, gastrointestinal and breast tumours. The pharmaceutical preparations included native human seminal plasma PSP⁹⁴ which could be administered in an appropriate dosage form, dosage quantity and dosage regimen to a patient suffering from prostate cancer. In another embodiment, the pharmaceutical preparation included a mixture of human seminal plasma PSP⁹⁴ and an anticancer drug which may be administered in an appropriate dosage form, dosage quantity and dosage regimen to a patient suffering from, for example gastrointestinal cancer.

PSP⁹⁴ sourced from human seminal fluid carries with it significant risk of contamination with infectious agents (e.g., HIV, hepatitis (a, b, or c), and other viruses and/or prions). Even with the use of harsh chemical treatment, total eradication of such agents cannot be guaranteed. Additionally, human

seminal fluid is found in limited supply, thus making bulk production of PSP⁹⁴ very difficult. Therefore, the acceptability of human or even xenogeneic sourced PSP⁹⁴ may be very difficult for both the regulatory authorities and the marketplace.

Therefore, the use of recombinant technology for producing PSP⁹⁴ would represent a significant advancement, as recombinant PSP⁹⁴ could be produced both free of pathogens and in an unlimited supply. Furthermore, the material would be homogeneous from a single lot source, avoiding batch to batch variation.

SUMMARY OF THE INVENTION

The present invention relates to the use of recombinant human rHu PSP⁹⁴ (SEQ ID NO: 2) for inhibiting the growth of prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian and other cancers of epithelial secretion, or benign prostate hyperplasia (BPH). Preferably, the rHu PSP⁹⁴ is used in a dosage range from about 500 picograms/kg/day to about 1 milligram/kg/day.

The recombinant human rHu PSP⁹⁴ has been expressed in *Pichia pastoris* (host strain GS115 (his4)), is non-glycosylated and has 10 cystein residues. The molecular weight of rHu PSP⁹⁴ was determined as 11.5 kDa, compared to 10.7 kDa for its native counterpart. An MTS cell proliferation assays showed that rHu PSP⁹⁴ could produce a dose dependent decrease in cell numbers of PC-3 human prostate adenocarcinoma cell line.

The invention also relates to the use of at least one peptide selected from the group consisting of the decapeptide depicted in SEQ ID NO: 3, the peptide analogue depicted in SEQ ID NO:4, the peptide analogue depicted in SEQ ID NO:5, and the

peptide analogue depicted in SEQ ID NO:6, or a mixture thereof, for inhibiting growth of prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian and other cancers of epithelial secretion, or benign prostate hyperplasia (BPH).

5 Preferably, the peptide is used in a dosage range from about 250 nanograms/kg/day to about 1 milligram/kg/day.

In another aspect of the invention there is provided a method for treating a patient with prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian and other cancers of epithelial secretion, or benign prostate hyperplasia (BPH), comprising administering to the patient a pharmaceutical preparation comprising a peptide selected from the group consisting of rHu PSP⁹⁴ (SEQ ID NO:2), the decapeptide depicted in SEQ ID NO: 3, the peptide analogue depicted in SEQ ID NO: 4, the peptide analogue depicted in SEQ ID NO: 5, and the peptide analogue depicted in SEQ ID NO:6.

The present invention also provides a composition for the use of inhibiting the growth tumours in patients suffering from prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian and other cancers of epithelial secretion as well as benign prostate hyperplasia (BPH). The composition comprises recombinant human rHu PSP⁹⁴ (SEQ ID NO: 2), preferably present in a dosage range of about 5 nanograms/kg/day to about 10 micrograms/kg/day. The composition preferably also includes an anticancer drug. A variety of suitable anticancer drugs are known in the art. Examples include, without limitation, mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin and daunomycin.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts mass spectrometry analysis of peptide (7-21) (SEQ ID NO: 4).

5

Figure 2 depicts mass spectrometry analysis of peptide (31-45) (SEQ ID NO:5).

Figure 3 depicts mass spectrometry analysis of peptide (76-94) (SEQ ID NO:6).

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Figures 4 - 6 are graphs depicting the inhibitory activity of the decapeptide of SEQ ID NO: 3 on in-vitro PC-3 cells.

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Figure 7 depicts a gel showing DNA fragmentation following treatment of PC-3 cells with the peptide depicted in SEQ ID NO:5.

20

Figure 8 is a graph depicting the results of an apoptosis assay with an ELISA plus kit following peptide treatment of ATCC-PC3 cells for 72 hours.

25

Figure 9 is a graph depicting growth of fibroblast cells when exposed to PSP⁹⁴ and its analogues in-vitro for 72 hours.

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Figure 10 is a graph depicting the effect of the peptides depicted in SEQ ID NOs:4 and 6 on the growth of ATCC-PC3 cells in-vitro at 72 hours.

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Figure 11 is a graph depicting the effect of peptides of the invention on growth of ATCC-PC3(OPTI) cells at 72 hours of exposure in-vitro.

Figures 12 and 13 are graphs depicting the results of studies of the anti-tumor efficacy validation of rPSP⁹⁴ against Mat Ly Lu (MLL) tumor implanted in nude mice.

5 Figure 14 is a graph depicting tumor growth reduction in rHu PSP⁹⁴-treated mice.

Figures 15 - 17 are graphs depicting tumor growth reduction in decapeptide (SEQ ID NO:3)-treated mice.

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DETAILED DESCRIPTION OF THE INVENTION

Various in-vivo and in-vitro experimental studies have
15 been carried out and are summarized herein below to determine the efficacy of concentrations of rHu PSP⁹⁴ (SEQ ID NO: 2) higher than native PSP⁹⁴ concentrations secreted by the diseased prostate as tumour suppressive agent for arresting or inhibiting the growth of prostatic adenocarcinoma. Studies have also been
20 carried out to determine the efficacy of decapeptide (SEQ ID NO:3) and synthetic peptide analogues of rHu PSP⁹⁴ (SEQ ID NO: 4, No. 5 and No.6) as tumour suppressive agents.

Studies were carried out using PC-3 human prostate
25 adenocarcinoma line which can be maintained both in vivo as a xenograft in nude mice and in vitro as a cell line. In addition, a rat Dunning Mat LyLu prostate tumour, which is a pre-eminent animal model for the study of CaP, was also used. The Dunning tumour is a fast growing, poorly differentiated, transplantable
30 tumour which can be maintained both in-vivo in the Copenhagen rat and in-vitro as a cell line.

The following examples are offered by way of illustration and not by way of limitation.

35

EXAMPLE 1**PREPARATION OF rHu PSP⁹⁴ (SEQ ID NO: 2)**

rHu PSP⁹⁴ was cloned and expressed in *Pichia pastoris*,
5 and then purified and characterized as follows.

Materials

DEAE-cellulose (DE52) was from Whatman, Fairfield, NJ
and the dialysis tubing from BioLynx (Pierce Inc.). Broad-range
10 molecular weight markers and Econo-pack columns fitted with flow
adapters were from Bio-Rad Labs Ltd CA. Pellicon device was from
Millipore, MA. Tris-HCl was obtained from ICN, and MES from
Sigma. Other reagents were obtained from standard commercial
sources and were of reagent grade. ECL detection kit was
15 purchased from Biolynx Canada and swine anti-rabbit IgG
alkaline-phosphatase conjugates from DAKO, Denmark. Rabbit
polyclonal antiserum against PSP94 was a gift from the late Dr.
A. Sheth. *Pichia* expression Kit version G was from Invitrogen,
Carlsbad, CA and the Non-Radioactive High Prime DIG labeling
20 kit® was from Boehringer Mannheim Indianapolis, IN. The MTS
assays were performed using Cell Titer Aqueous Non radioactive
cell proliferation assay kit from Promega Madison, WI. MRX
microtiter plate reader was from Dynex technologies, Chantilly,
VA. All primers were synthesized by Procyon Biopharma Inc.
25 London, ON.

Cell lines and Cell cultures

P. pastoris host strain GS115 (*his4*) and all the
Pichia related products were obtained from Invitrogen. PC3
30 (ATCC-# CRL 1435) cell line was from the American Type Cell
Culture (ATCC) and maintained in OPTI MEM media with 10 % FBS.
All cell culture products were obtained from GIBCO BRL.

Cloning and Expression

TA cloning vector (pCR™ 2.1) containing human PSP94 cDNA with 20 a.a. leader sequence used previously (9) (Baijal et. al. 1999) was modified without its leader sequence to amplify human PSP94. The primers for the PCR reaction were designed to contain EcoRI restriction sites at either end. The 5' primer used was 5'-GGG AAG [↓]AAT TCT CAT GCT ATT TCA TA-3' and the 3' primer, 5'-TGG ATA TCT GCA GAA TT[↓]C GGC-3'. The EcoRI cleavage signal has been designated with [↓] and the +1 start site for PSP94 (Ser), has been underlined. The subcloning of the PSP94 insert was done in pPIC9 vector (Invitrogen), which contained EcoRI restriction site at the 5' end, about 5-8 amino acids away from the cleavage site that removes the signal sequence of the PSP94 insert. The amplification of the product was done by PCR, using BM Expand™ High Fidelity PCR System.

The PCR included 1 cycle for 12' @ 94 C, 25 cycles for 1' @ 94 C followed by 1' @ 55 C, 1' @ 72 C and final 10' @ 72 C. The product was run on 1.5 % agarose gel and appropriate bands isolated using Pharmacia Sephaglass Kit (Bandprep). The EcoRI enzyme was used for the restriction digestions of both the plasmid and the PCR products followed by ligation and transformations, using DH5α cells. The isolated clones were selected for by ampicillin resistance and inserts were identified by restriction mappings. The constructs were sequenced (Robart's sequencing service, London ON) to identify PSP94 insert with correct orientation and reading frame as well scrutinized for point mutations.

To follow the time course of expression and to determine the optimum time for harvesting secreted PSP94, daily aliquots of the expression medium were removed beginning with the first day of induction, until the time of harvesting (96 h). Expression of the protein was tracked by SDS PAGE with 15 % gel,

followed by Western blot analysis using polyclonal antibody raised against native PSP94.

Screening for High Expressing Clones

5 About 100 clones were picked and grown into 2 ml culture media to OD₆₀₀ of ~6. Total DNA was isolated for rapid dot blot technique to detect multiple integration by Southern blot analysis to screen for high PSP94 expressing clones. 200ul of each culture specimens were denatured and blotted to a
10 positively charged nylon membrane, placed in the dot blot apparatus (in duplicate) and air-dried. The membrane was soaked between two sheets of Whatman 3MM paper for 15 min in a solution with 50 mM EDTA, 2.5 % BME, pH 9, followed by 24 hours at 37 C with 1 mg/ml Zymolyase 100T, 5min in 0.1 N NaOH, 1.5 M NaCl,
15 0.015M sodium citrate pH 7 and 2 x 5 min in 2xSSC. Finally the membrane was baked at 80 C for 45 min and UV exposed for 15min. Human PSP94 cDNA probe was labeled with the non radioactive High Prime DIG labeling kit® (Boehringer Mannheim) and was used for hybridization (9). All DIG labeling procedures were performed
20 according to the manufacturer's protocol and the hybridization signals were detected on the Hyper film-ECL (Amersham Life Science Inc. Arlington Hts, IL).

Hybridization with DIG labeled cDNA probe (25ng/ul)
25 was done for 2 days at 42 C in high SDS buffer (SDS 7 %; formamide, 50%; 5 X SSC; 50 mM sodium phosphate, pH 7.0; N-lauroyl-sarcosine, 0.1 % (w/v)) and blocking reagent, 2 %. CSPD® (Boehringer Mannheim) was used as the chemiluminescence substrate. The clone with highest signal intensity was used for
30 all flasks shaken cultures.

Optimization of the Expression of the Protein in the Flask Shaken Cultures

A single clone containing the PSP94 construct was selected for the optimum expression of the protein. 25ml of BMG medium was grown until the OD₆₀₀ measured between 2 and 6. The flask shaken cultures in 1 liter volumes of BMG media were inoculated with 25ml culture and incubated in Baffled Erlenmeyer flasks for growth phase, until OD₆₀₀ reached approximately 2.0 to 6.0. The culture was centrifuged for 15min at 2500 X g and the pellet collected. The growth in the induction phase was then carried out in BMM media by inoculating it with the collected cell pellet in Baffled flasks and grown for 6 days, as described by Invitrogen. The volume of BMM varied based on the amount of pellet. Five ml of 100% methanol was added per litre of culture, each day, around the same time, to a final concentration to 0.1 %. The plasmid without the insert served as a negative control.

To determine the optimum time for harvesting secreted PSP94, aliquots were monitored every 24 hrs for 6 days, beginning with the first day of induction. The levels of PSP94 protein were determined by measuring the cell densities at OD₆₀₀ and by 15 % Comassie stained SDS PAGE and Western blot analysis, using polyclonal antibody against native PSP94.

Sample Preparation

The specimen from the flask shaken culture with the highest rPSP94 expression, post induction growth (96 hr), was centrifuged at 2500 X g for 20min. The supernatant was filtered through 0.8µm filter and concentrated approximately 10-fold using the Pellicon unit (Millipore). The specimen was dialyzed against 0.05 mM Tris HCl buffer, pH 8.0, using a Mr 3500 cut-off tubing. The final supernatant was tested by SDS PAGE and Western blot analysis and further used for purification.

Culture Conditions for Fermentation

The fermentation was carried out at the Institute for Biological Sciences, National Research Council Ottawa ON Canada, following the manufacturer's protocol. The fermentation

5 procedure was initiated by inoculating 7.5-liter medium with 625 ml of starter flask shaken culture material. The growth phase culture was carried out for 2 days in BMG medium until the OD₆₀₀ reached approx. 0.5. The initial growth phase was terminated and induction initiated by the addition of 100 % methanol, as
10 described by inVitrogen. The culture was finally harvested at 95 hr after being on methanol for 67 hrs. The final volume of the culture was approximately 13.5 lit.

Sample Preparation using Fermentation Culture

15 The large cell mass was removed by centrifugation and was performed at NRC. The cell free medium collected (9 lit) was further clarified by 0.2 u filtration Pellicon unit. The final 8.5 lit containing secreted rPSP94 was tested for rPSP94 expression and saved at -20 C for isolation and purification of
20 the protein.

Protein Estimation

The protein in the culture supernatants of the flask shaken and the fermentation was based on band intensities of
25 various amounts of the supernatant samples on the SDS PAGE gels. Standard curve were constructed by electrophoresis of increasing predetermined quantities of pure lyophilized PSP94 on the same gel. The initial estimate for rPSP94 at each step of purification was determined by OD₂₈₀. Quantification of protein
30 at the final steps of purification was done by BCA method, using bovine serum albumin as standard.

Lyophilization

Samples of purified rPSP94 were dialyzed against deionized water using Mr 3000 cut-off membrane and lyophilized.

5 SDS gel electrophoresis

SDS gel electrophoresis was performed using 15% acrylamide separating and 5% stacking gels, containing 0.1% SDS according to Laemmli under reducing conditions. Broad-range molecular weight markers were used for the estimation of
10 molecular weights of the protein. Protein bands were stained with Coomassie Brilliant Blue R-250.

Western Blotting

For immunoblotting, Mini Trans-Blot Electrophoretic
15 Transfer Cell (Bio Rad) was used with Hi bond-C supper membrane (Amersham) and 85mm blotting papers. Protein samples, 0.4 µg each, run on SDS PAGE gel, as described earlier, were transferred to the membrane using transfer buffer (25 mM Tris, 192 mM Glycine, pH 8.3 and 20 % methanol) at 200 mA° for 2hrs at
20 4° C. Membranes were blocked O/N by incubation in 2% (w/v) non-fat dry milk (skim milk) dissolved in 500 mM NaCl, 20 mM Tris-HCl, pH 7.5 (TBS) at room temp (RT). Immunoblots were washed three-times with TBS containing 0.02% (v/v) Tween 20 (TTBS). The blot was subsequently incubated with anti PSP94 (1:2000
25 dilution) in TTBS with 2% skim milk for 2 hrs at RT. Membranes were washed thrice with TTBS, 5min each, incubated with secondary antibody, swine anti rabbit antibody HRP conjugated ((1:5000) in TTBS at RT and washed thrice with 0.02% TTBS 5 minutes each. Blots were developed using the ECL detection
30 system, as per manufacturer's instructions, using the Super Signal Substrate, and exposed to a Hyperfilm ECL from Amersham LS for 5 to 20 sec. Pre-stained molecular weight markers were used for the estimation of molecular weights of the protein.

DE52 Column Chromatography

After removal of *P. pastoris* cells from the fermentation culture, PSP94 containing supernatant was concentrated ~ ten fold, dialyzed and subjected to anion exchange chromatography. A column of DE52 with a bed volume ~ 40ml (2.5 (id) X 8 cm (h) cm) was equilibrated with 0.05 M Tris-HCl, pH 8.0. The sample (25 ml) containing 15-20 mg PSP94 protein was applied to the DE52 column at a flow rate of 1 ml/min. Impurities from the column were removed by washing it with 40-50 ml of the equilibrating buffer, and monitoring the A₂₈₀. This step was followed by 100-150 ml of 0.05 M Tris-HCl, pH 6.5 and most importantly till the pH of the wash was ~6.5. The column was further washed with 100- 150 ml of 0.05 M MES-acetate buffer, pH 6.5, until the A₂₈₀ value approached zero. Finally rPSP94 was eluted from the column with 0.05 M MES-acetate buffer, pH 5.0. Peak fractions were identified by A₂₈₀ determination, followed by SDS PAGE and Western blot analysis as described above. Fractions with high A₂₈₀ values (0.5 to 1.8) were pooled and dialyzed against water/PBS for storage at -20 C and/or lyophilization.

Amino acid Composition

The amino acid analysis of the DE52 purified flask shaken and the fermentation specimens was carried out at the Louisiana State University Medical Core Laboratory. The Perkin Elmer Biosystems Derivatizer-Analysis system was used with Spheri-5 PTC C-18 5u column and UV detection at OD₂₅₄.

Mass Spectral analysis

Purified samples from the flask shaken and the fermentation cultures were analyzed using the PerSeptive Biosystems (Framingham, MA), with Voyager-DE MALDI-TOF mass spectrometer using 337 nm light from a nitrogen laser at the Louisiana State University Medical Core Laboratory. About 50

scans were averaged for each analysis. A sample from the native PSP94 was also analyzed under similar conditions for comparison.

5

EXAMPLE 2**EFFECT OF rHu PSP⁹⁴ ON IN-VITRO PC-3 CELLS
(MTS ASSAY)**

The biological activity of the rHu PSP⁹⁴ was determined by its growth inhibitory effects on the human prostate cancer cells PC-3. The cell proliferation was monitored on PC-3 cells using the MTS/PMS kit (Promega), which primarily measures the mitochondrial activity of live cells. In addition, a visual observation of the cells was also done to check the cell morphology, which could also result in inhibition of cell growth. These results are summarized in Table 1. The following conditions for MTS assay were used:

PC-3 (ATCC, Lot AT06), Passage Number $n \geq 70$, Cell line adapted to grow in serum-free OPTI-MEM. Continuous exposure for 72 hrs without changing medium (adding PSP⁹⁴ at 2X concentration directly to wells and diluting it 1:2 to 1X to minimize cell manipulation and avoid detachment). Evaluation of rHu PSP⁹⁴ demonstrated a substantial reduction in cell numbers ranging from 37-57% at 80 and 120 $\mu\text{g/ml}$ concentrations, respectively in 3 out of 4 experiments (Table 1). Results of Trypan Blue Exclusion Test demonstrated 62% inhibition of cell viability at 80 $\mu\text{g/ml}$.

TABLE 1: Effect of rHu PSP⁹⁴ on in vitro PC-3 cells (MTS assay)

Reference:
 /Monthly Report June
 Activity 2000)

Experiment #	rHu PSP ⁹⁴	% Viability (control = 100%) at µg/ml				
		40	60	80	120	
079-140	00-133F	72	78	58	43	
079-105	"	63	63	63	68	
079-120	"	95	85	78	ND	
079-153	"	100	52	62	60	
079-140	99.09.08	100	98	90	52	
rHu PSP ⁹⁴		% Viability (control = 100%) at µg/ml				
		5	10	20	40	80
00-133F		98	84	78	70	55
"		92	95	80	71	59
"		89	69	79	68	65

5

EXAMPLE 3

**EFFECT OF rHu PSP⁹⁴ ON IN-VITRO PC-3 CELLS
 ([³H]-THYMIDINE UPTAKE ASSAY)**

10

The vitro rHu PSP⁹⁴ efficacy validation was assessed in the [³H]-Thymidine Uptake assay where PC-3 cells are exposed to various concentrations of rHu PSP⁹⁴ for 72 hours and during the final 16 hrs of incubation the cells are pulsed with 1 µCi of [³H]-Thymidine. These results are summarized in Table 2.

15

Evaluation of rHu PSP⁹⁴ demonstrated a 65% reduction in the %age of cells incorporating [³H]-thymidine following treatment at 80 µg/ml for 72hrs. For comparison of sensitivities between [³H]-Thymidine Uptake and the MTS assays an additional plate was set aside for MTS assay evaluation and treated in parallel with the same lot of rHu PSP⁹⁴. The results demonstrated a 35% reduction in cell viability (65% cells remaining viable) following treatment at 80 µg/ml, indicating that the [³H]-Thymidine Uptake assay might be more sensitive than the MTS assay.

20

TABLE 2: Effect of rHu PSP94 on in vitro PC-3 cells
([³H]-Thymidine uptake assay)

Experiment #	PSP ⁹⁴	[³ H]-Thymidine Uptake (% of control) at µg/ml				
		5	10	20	40	80
1	rHu PSP ⁹⁴ (00-133F)	94	101	98	79	35
1	n PSP ⁹⁴ 99413S (B2)	97	98	100	98	77

5

EXAMPLE 4

EFFECT OF DECAPEPTIDE AND OTHER PEPTIDE ANALOGUES ON IN-VITRO PC-3 CELLS

10 The synthetic decapeptide (SEQ ID NO: 3) has been
shown herein to mimic the biological action of native nPSP⁹⁴
(SEQ ID NO: 1) and therefore its effect on the PC-3 cells was
studied in clonogenicity assay. Referring to Figures 4-6, the
decapeptide (SEQ ID NO: 3) has a similar inhibitory action as
15 nPSP⁹⁴ (SEQ ID NO: 1) on in-vitro PC-3 cells was studied.
Specifically, a 40% colony count inhibition was observed with 1
ug/ml of the decapeptide (SEQ ID NO: 2) leading to a maximum of
60% inhibition at 10 µg/ml.

20

EXAMPLE 5

DNA FRAGMENTATION ASSAY

DNA fragmentation assay measures the extent of
apoptosis by the presence of DNA ladder on 1.2% Agarose gel
25 following exposure of PC-3 to various concentrations of the
peptide analogues. Peptide analogues used: peptide 7-21 (SEQ ID
NO: 4), peptide 31-45 (SEQ ID NO:5) and peptide 76-94 (SEQ ID
NO:6). Duration of treatment 72 hrs. DNA was isolated and run

on 1.2% Agarose gel. Results demonstrated that all the peptides tested produced a DNA laddering effect characteristic of apoptosis. This effect was especially evident following treatment with peptide 31-45 (SEQ ID NO: 5) (Figure 7).

5

EXAMPLE 6

APOPTOSIS ASSAY BY ELISA PLUS

10 The three peptides analogues (SEQ ID NO: 4, No.5 and No. 6) and native PSP⁹⁴ as a positive control were tested in ELISA plus assay to measure cell death through apoptosis. The PC-3 cells were treated with various concentrations of peptides for 72 hrs. It was observed that a dose dependent increase in
15 the apoptotic cell death was noticed in all the 3 peptides analogues (SEQ ID NO: 4, No.5 and No. 6). The middle portion peptide (SEQ ID NO: 5) was more potent at 90 μ m concentration (Figure 8). Apoptosis assay was done as per instructions in the ApopTag kit (Boerenger Mannheim).

20

EXAMPLE 7

INHIBITION OF CELL-GROWTH BY PSP⁹⁴ PEPTIDE ANALOGUES

25 The biological activity of the peptide analogues (SEQ ID NO: 4, 5 and 6) was determined by their growth inhibitory effects on the human prostate cancer cells PC-3. The cell proliferation was monitored on PC-3 cells and control normal fibroblast using the MTS/PMS kit (Promega), which primarily
30 measures the mitochondrial activity of live cells. In addition, a visual observation of the cells was also done to check the cell morphology, which could also result in inhibition of cell growth. These results are show in Figures 9 and 10. No cell inhibitory effect was observed against fibroblast cells
35 following incubation with various peptide concentrations for 72

hrs (Figure 9 and Figure 11) whereas significant growth inhibition was observed especially with middle portion peptide fragment 31-45 (SEQ ID NO: 5) as well as the amino and carboxy terminus peptide fragments (SEQ ID NOs: 4 and 6, respectively) (Figure 10).

EXAMPLES 8 & 9

IN-VIVO EXPERIMENTS (Figures 12 & 13)

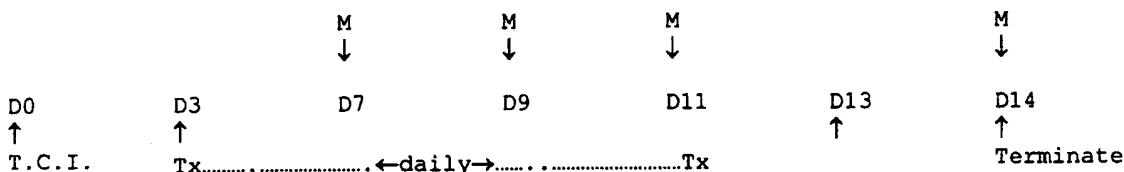
(Study MLL-1)

Anti-tumor Efficacy Validation of rPSP⁹⁴ against Mat Ly Lu (MLL) Tumor Implanted in **Nude Mice**.
(Protocol based on S. Garde et al.; The Prostate, 22: 225-233, 1993 "Effect of Prostatic Inhibin Peptide (PIP) on Prostate Cancer Cell Growth In Vitro and In Vivo")

Groups (n = 8 mice / group):

- **Group 1:** Negative control PBS s.c.
- **Group 2:** Positive control Doxorubicin @ 5mg/kg i.v. single bolus on Day 3
- **Group 3:** rPSP⁹⁴ Batch # 00-133F @ 1µg/kg s.c.
- **Group 4:** rPSP⁹⁴ Batch # 00-133F @ 10µg/kg s.c.
- **Group 5:** rPSP⁹⁴ Batch # 00-133F @ 100µg/kg s.c.

Schematic:



(Study MLL-2)

Anti-tumor Efficacy Validation of rPSP⁹⁴ against Mat Ly Lu (MLL) Tumor Implanted in **SCID Mice**.

(Protocol based on S. Garde et al.; The Prostate, 22: 225-233, 1993 "Effect of Prostatic Inhibin Peptide (PIP) on Prostate Cancer Cell Growth In Vitro and In Vivo")

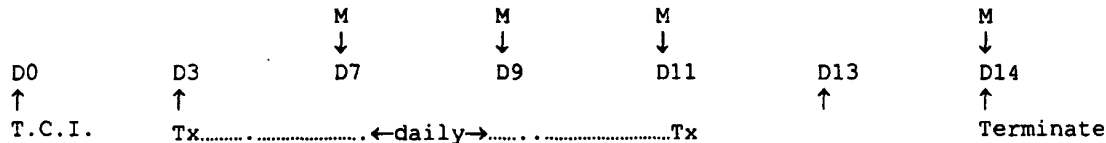
Groups (n = 8 mice / group):

- **Group 1:** Negative control PBS s.c.

Group 2: Positive control Doxorubicin @ 5mg/kg i.v. single bolus on Day 3

- **Group 3:** rPSP⁹⁴ Batch # 00-133F @ 1µg/kg s.c.
- **Group 4:** rPSP⁹⁴ Batch # 00-133F @ 10µg/kg s.c.
- **Group 5:** rPSP⁹⁴ Batch # 00-133F @ 100µg/kg s.c.

Schematic:



Conclusions For Examples 8 & 9:

- Difference in tumor size and growth in Nude vs SCID. The tumors were smaller in SCID mice. Maybe due to some specific factors controlling tumor growth.
- Good tumor reduction with Doxorubicin (good positive control):
 - Tumor Weight Reduction in Nude mice (MLL-1): **48% p=0.006**
 - Tumor Weight Reduction in SCID mice (MLL-2): **82% p=0.002**
- Significant tumor reduction with rPSP⁹⁴ @ 1µg/kg:
 - Tumor Weight Reduction in Nude mice (MLL-1): **26% p=0.042**
 - Tumor Weight Reduction in SCID mice (MLL-2): **65% p=0.010**

EXAMPLE 10 (Figure 14)

IN-VIVO EXPERIMENT USING PC-3 CELL LINE

PC3 human prostate tumor (ATCC 1435) obtained from ATCC was implanted into nude mice and tumor growth monitored for approximately 18 days. The PC-3 cells were injected once subcutaneously into the shoulder. Once tumor growth has been established (50 mm³ volume) the treatment with rHu PSP⁹⁴ (SEQ ID

NO: 2) begun at qldx14 s.c. based on the following assigned treatment groups (n= 8 per group):

Treatment Groups	Test and control Articles	Dose Level* (µg/kg)	Dos Conc. (µg/mg)	No of Animal
1 (Neg Control)	PBS	0	0	8
2 (Pos Control)	Doxorubicin	5000**	2500	8
3	rHu PSP ⁹⁴	1	0.5	8
4	rHu PSP ⁹⁴	10	5	8
5	rHu PSP ⁹⁴	100	50	8
6	rHu PSP ⁹⁴	1000	500	8

Results of this experiment (Figure 14) demonstrated tumor growth reduction in the rHu PSP⁹⁴ treated group of mice at a dosage level of 1 µg/kg body weight per day similar to that of Doxorubicin (given at 5 mg/kg) a chemotherapeutic agent used as reference gold standard.

EXAMPLE 11 (Figures 15-17)

IN-VIVO EXPERIMENT USING PC-3 CELL LINE

PC3 human prostate tumor (ATCC 1435) obtained from ATCC was implanted bilaterally into nude mice and tumor growth monitored for approximately 18 days. The PC-3 cells were injected once subcutaneously into each flank. Once tumor growth has been established (0.25-0.50 cc volume) the treatment with decapeptide PB102 (SEQ ID NO:3), native PSP⁹⁴ (PB100) (Sequence ID. 1) and control irrelevant decapeptide PB111

begun at qldx14 s.c. based on the following randomly assigned treatment groups (n=4 per group):

Treatment Groups	Test and control Articles	Dose Level* (µg/kg)	Dose Conc. (µg/mg)	No of Animal
1 (Neg Control)	PBS	0	0	4
3	PB102 (decapeptide)	1	0.5	4
4	PB102 (decapeptide)	10	5	4
5	PB102 (decapeptide)	100	50	4
6	PB102 (decapeptide)	1000	500	4
7	PB100 (nPSP ⁹⁴)	1	0.5	4
8	PB100 (nPSP ⁹⁴)	10	5	4
10	PB100 (nPSP ⁹⁴)	100	50	4
11	PB100 (nPSP ⁹⁴)	1000	500	4
12	PB111 (control)	1	0.5	4
13	PB111 (control)	10	5	4
14	PB111 (control)	100	50	4
15	PB111 (control)	1000	500	4

Results of this study (Figures 15-17) demonstrated significant (p<0.05) tumor growth reduction in the decapeptide PB102- treated group of mice at a dosage level of 10 µg/kg body weight per day.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. The citation of any publication is for its disclosure prior to the

filing date and should not be construed as an admission that the present invention is not entitled to antedate such publication by virtue of prior invention.

5 Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it is readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made
10 thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

5 SEQ ID No: 1
(nPSP94 sequence):

NH
SCYFIPNEGVPGDSTRKCMDLKGNKHPINSEWQTDNCETCTCYETEISCCTLVSTPVGYDKDNC
10 QRIFKKEDCKYIVVEKKDPKKTCSVSEWII COOH

15 SEQ ID NO: 2
(rPSP sequence):

NH
EAEAYVEFSCYFIPNEGVPGDSTRKCMDLKGNKHPINSEWQTDNCETCTCYETEISCCTLVSTP
VGYDKDNCQRIFKKEDCKYIVVEKKDPKKTCSVSEWII COOH

20 SEQ ID NO: 3
(decapeptide)

25 Tyr-Thr-Cys-Ser-Val-Ser-Glu-Pro-Gly-Ile

30 SEQ ID NO: 4
(N-terminus peptide 7-21)

NH
Asn-Glu-Gly-Val-Pro-Gly-Asp-Ser-Thr-Arg-Lys-Cys-Met-Asp-Leu

35 SEQ ID NO: 5
(middle portion peptide 31-45)

40 Glu-Trp-Gln-Thr-Asp-Asn-Cys-Glu-Thr-Cys-Thr-Cys-Tyr-Glu-Thr

45 SEQ ID NO: 6
(carboxy-terminus 76-94)

Ile-Val-Val-Glu-Lys-Lys-Asp-Pro-Lys-Lys-Thr-Cys-Ser-Val-Ser-Glu-
Trp-Ile-Ile COOH

50

5 CLAIMS:

1. Use of human rHu PSP⁹⁴ (SEQ ID NO: 2) for inhibiting growth of prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH), wherein the human rHu PSP⁹⁴ is used in a dosage range from about 500 picograms/kg/day to about 1 milligrams/kg/day.
2. The use of human rHu PSP⁹⁴ (SEQ ID NO: 2) according to claim 1 wherein the human rHu PSP⁹⁴ is used in a dosage range from about 5 nanograms/kg/day to about 10 micrograms/kg/day.
3. The use of human rHu PSP⁹⁴ (SEQ ID NO: 2) according to claim 1 wherein the human rHu PSP⁹⁴ is used in a mixture comprising the human rHu PSP⁹⁴ (SEQ ID NO: 2) and a pharmaceutically acceptable carrier.
4. The use of human rHu PSP⁹⁴ (SEQ ID NO: 2) according to claim 3 wherein the human rHu PSP⁹⁴ is used in a dosage range from about 5 nanograms/kg/day to about 500 nanograms/kg/day.
5. The use of human rHu PSP⁹⁴ according to claim 3 wherein the pharmaceutically acceptable carrier includes time release encapsulation means for effecting continual dosing of the human rHu PSP⁹⁴.
6. The use of human rHu PSP⁹⁴ according to claim 5 wherein the time release means comprises a liposome.
7. The use of human rHu PSP⁹⁴ according to claim 5 wherein the time release means comprises a polysaccharide.

5 8. The use of human rHu PSP⁹⁴ according to claim 1 wherein human rHu PSP⁹⁴ is used in a mixture including an anticancer drug.

9. The use of humans rHu PSP⁹⁴ according to claim 8
10 wherein the anticancer drug is selected from the group consisting of mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin and daunomycin.

10. Use of at least one peptide selected from the group
15 consisting of the synthetic decapeptide depicted in SEQ ID NO:3, the peptide analogue depicted in SEQ ID NO:4, the peptide analogue depicted in SEQ ID NO:5, and the peptide analogue depicted in SEQ ID NO:6, for inhibiting growth of prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial,
20 ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH), wherein the peptide is used in a dosage range from about 250 nanograms/kg/day to about 1 milligrams/kg/day.

25 11. A method for treating a patient with prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH), the method comprising administering to the patient a pharmaceutical preparation comprising at least
30 one peptide selected from the group consisting of human rHu PSP⁹⁴ (SEQ ID NO:2), the decapeptide depicted in SEQ ID NO:3, the peptide analogue depicted in SEQ ID NO:4, the peptide analogue depicted in SEQ ID NO:5, and the peptide analogue depicted in SEQ ID NO:6).

35 12. The method of claim 11 wherein the pharmaceutical preparation includes an anticancer drug.

- 5 13. The method of claim 12 wherein the anticancer drug is selected from the group consisting of mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin and daunomycin.
- 10 14. The method according to claim 13 wherein the peptide is human rHu PSP⁹⁴ (SEQ ID NO: 2) and wherein it is administered in a dosage range from about 25 picograms/kg/day to about 1 milligrams/kg/day.
- 15 15. The method according to claim 13 wherein the human rHu PSP⁹⁴ is administered in a mixture including an anticancer drug, wherein the anticancer drug is selected from the group consisting of mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin and daunomycin.
- 20 16. The method according to claim 14 wherein the human rHu PSP⁹⁴ is administered in a dosage range from about 5 nanograms/kg/day to about 10 micrograms/kg/day.
- 25 17. The method according to claim 16 wherein the human rHu PSP⁹⁴ is administered in a mixture including an anticancer drug selected from the group consisting of mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin and daunomycin.
- 30 18. The method according to claim 16 wherein the human rHu PSP⁹⁴ (SEQ ID NO: 2) is used in a mixture including a pharmaceutically acceptable carrier.
- 35 19. The method according to claim 18 wherein the pharmaceutically acceptable carrier includes time release encapsulation means for effecting continual dosing of the human rHu PSP⁹⁴.

5

20. The method according to claim 19 wherein the time release encapsulation means comprises a liposome.

21. The method according to claim 19 wherein the time release encapsulation means comprises a polysaccharide.

22. The method according to claim 11 wherein the peptide is administered in a dosage range of about 25 nanograms/kg/day to about 50 micrograms/kg/day.

15

23. The method according to claim 22 wherein the pharmaceutically acceptable carrier includes time release encapsulation means for effecting continual dosing of the peptide.

20

24. A composition for inhibiting the growth tumours in patients suffering from prostatic adenocarcinoma, stomach cancer, breast cancer, endometrial, ovarian or other cancers of epithelial secretion, or benign prostate hyperplasia (BPH), comprising:

25

a) Human rHu PSP⁹⁴ (SEQ ID NO: 2) present in a dosage range of about 5 nanograms/kg/day to about 10 micrograms/kg/day; and

30

b) an anticancer drug.

25. A composition according to claim 24 wherein said anticancer drug is selected from the group consisting of mitomycin, idarubicin, cisplatin, 5-fluoro-uracil, methotrexate, adriamycin and daunomycin.

35

5 26. A composition according to claim 24 including a
pharmaceutically acceptable carrier.

27. A composition according to claim 24 including a
pharmaceutically acceptable carrier includes time release
10 encapsulation means for effecting continual dosing of the
composition.

28. A method for treating patients with diseases
characterized by elevated levels of FSH comprising administering
15 a pharmaceutical preparation in an appropriate dosage form, the
pharmaceutical preparation comprising at least one peptide
selected from the group consisting of human recombinant PSP⁹⁴
(SEQ ID NO: 2), the decapeptide depicted in SEQ ID NO: 3, the
peptide depicted in SEQ ID NO:4, the peptide depicted in SEQ ID
20 NO:5, and the peptide depicted in SEQ ID NO:6.

Procyon

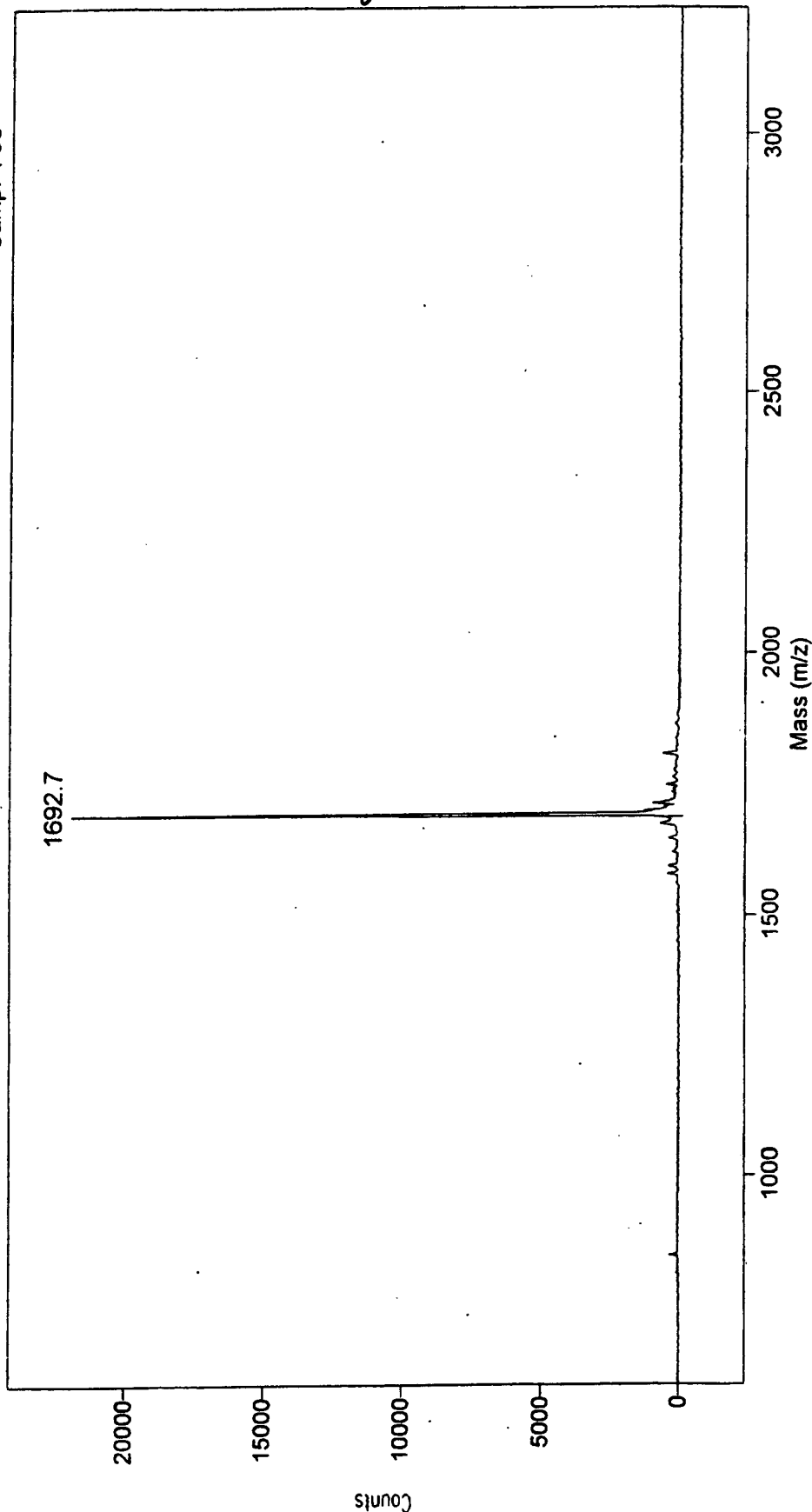
(SEQ ID NO:4)

Sample: PSPa(7-21)

MW= 1691.5

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Sampl : 98



Comment:

Method: LDE1008A

Mode: Linear

Accelerating Voltage: 20000

Grid Voltage: 94.000 %

Guide Wire Voltage: 0.030 %

Negative Ions: OFF

Laser : 1860

Scans Averaged: 12

Pressure: 7.15e-07

Low Mass Gate: OFF

Timed Ion Selector:

Mirror Ratio:

PSD Mirror Ratio:

Fig. 2

Procyon (SEQ ID NO: 5)

Sample: PSI'a(31-45)

MW= 2034.9

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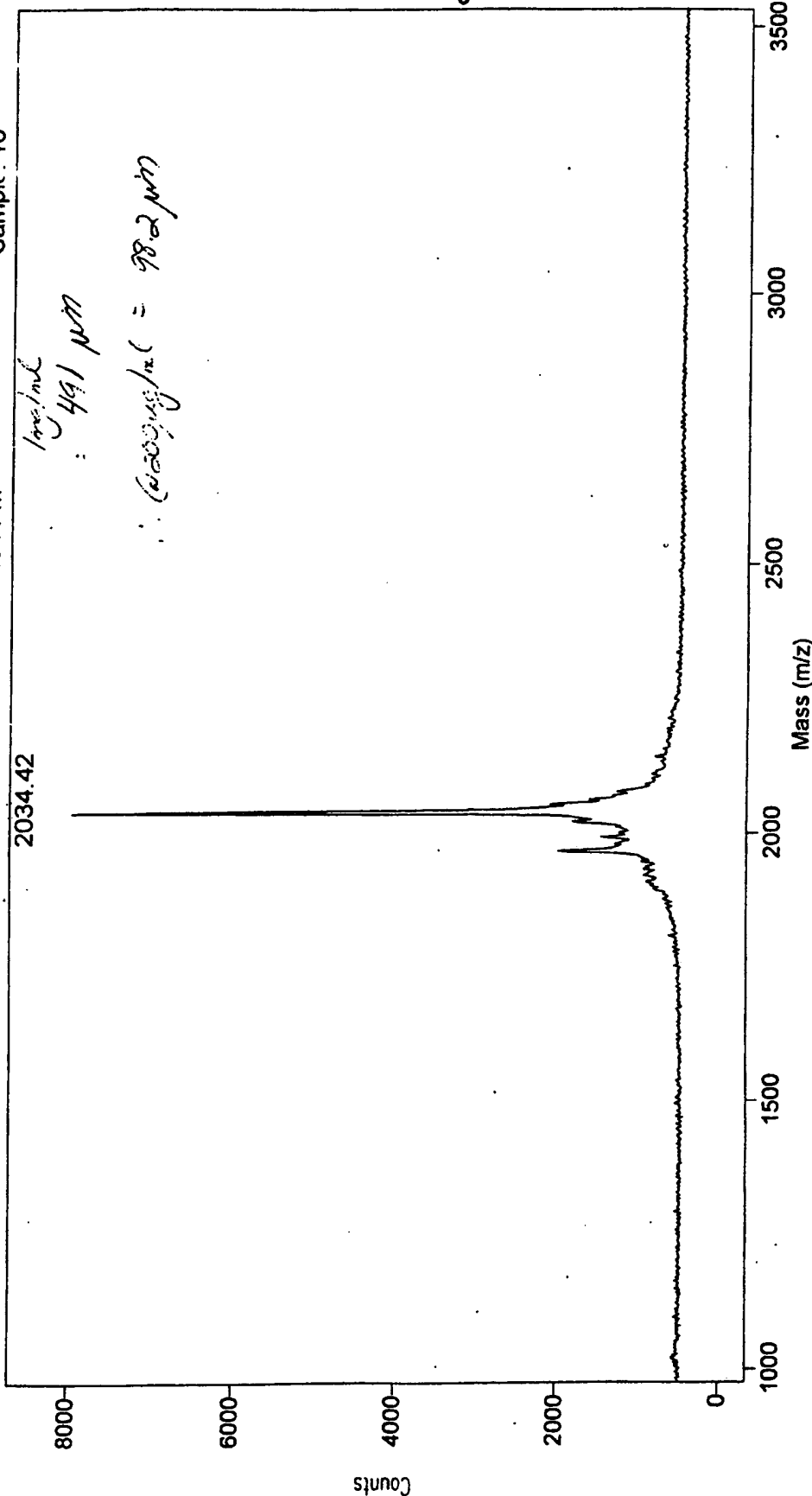
Sample: 16

2034.42

1 mg/ml

: 491 μ M

\therefore 6200 ng/ml = 98.2 μ M



Comment:

Method: LDE1008B

Mode: Linear

Accelerating Voltage: 20000

Grid Voltage: 94.000 %

Guide Wire Voltage: 0.060 %

Negative Ions: ON

Laser : 1790

Scans Averaged: 18

Pressure: 2.39e-07

Low Mass Gate: OFF

Timed Ion Selector:

Mirror Ratio:

PSD Mirror Ratio:

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Fig. 3

Procyon (seq 10 no: 5)

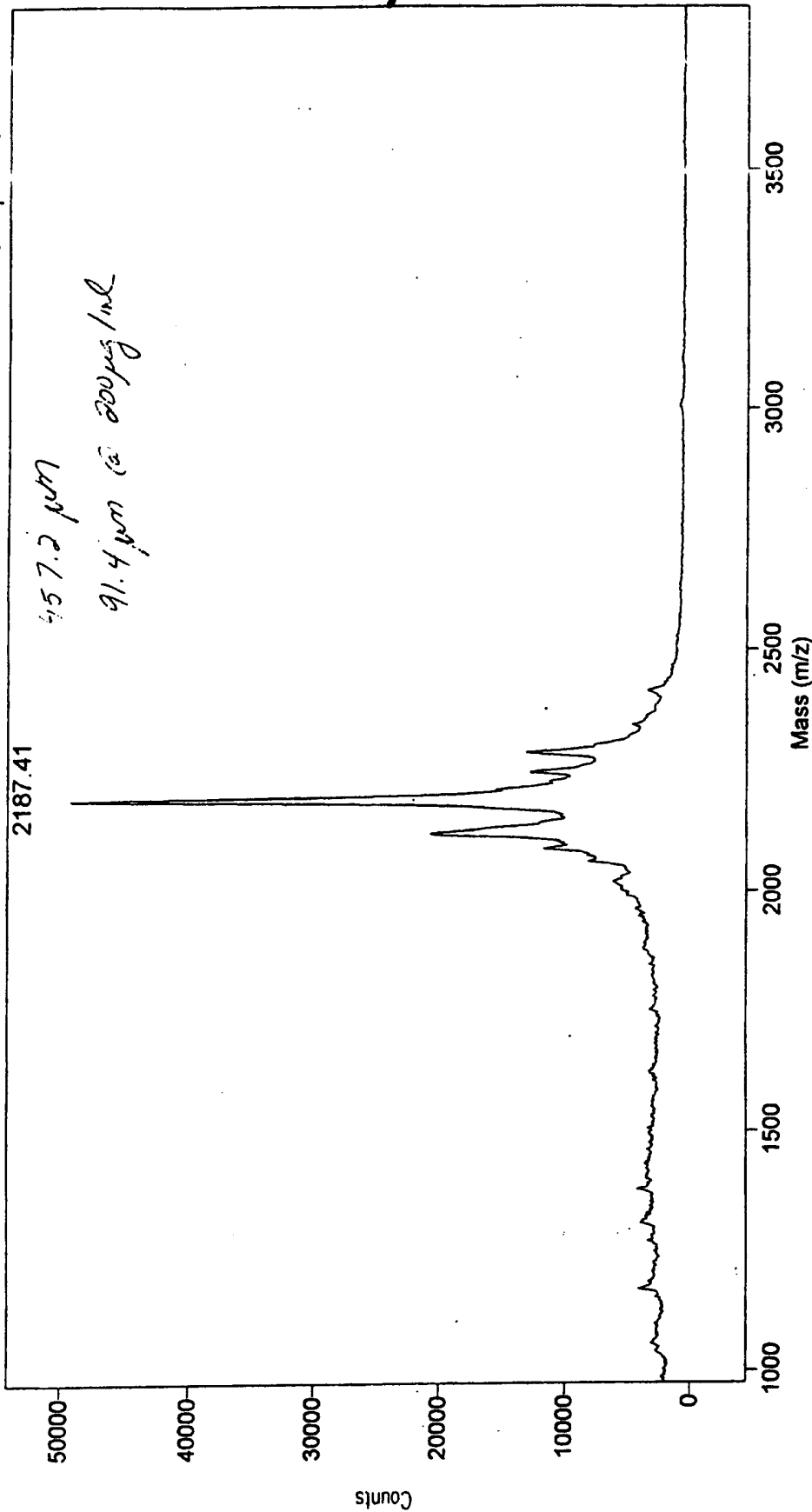
Sample: PSI'a(76-94)

MW= 2187.2

N.B. This Sub is detected Top & Top (at 3.1 min + 2 min) in red

Sample: 45

File # 1 : C:\VOYAGER\PROCYON\SMOOTH.MS Collected: 9/22/98 11:01 AM



Comment:

Method: LDE1008B

Mode: Linear

Accelerating Voltage: 20000

Grid Voltage: 94,000 %

Guide Wire Voltage: 0.060 %

Negative Ions: OFF

Laser : 1920

Scans Averaged: 27

Pressure: 4.40e-07

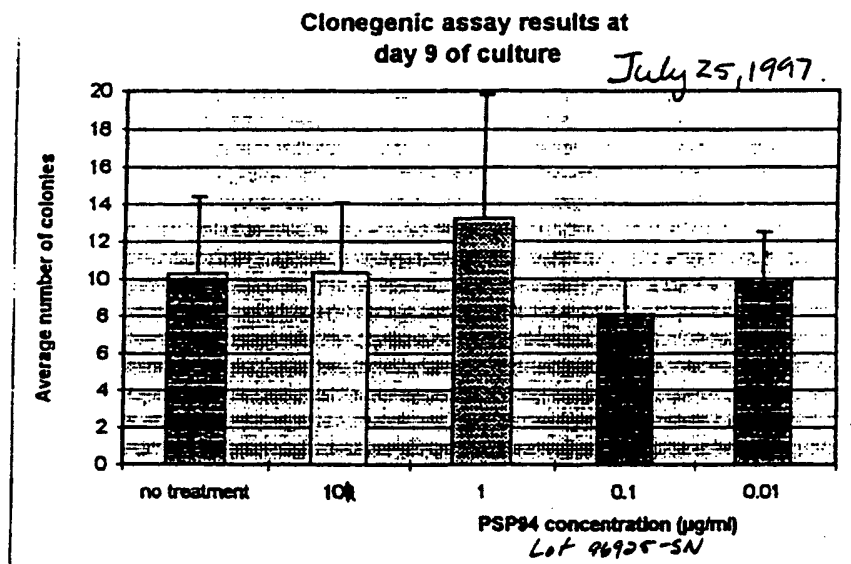
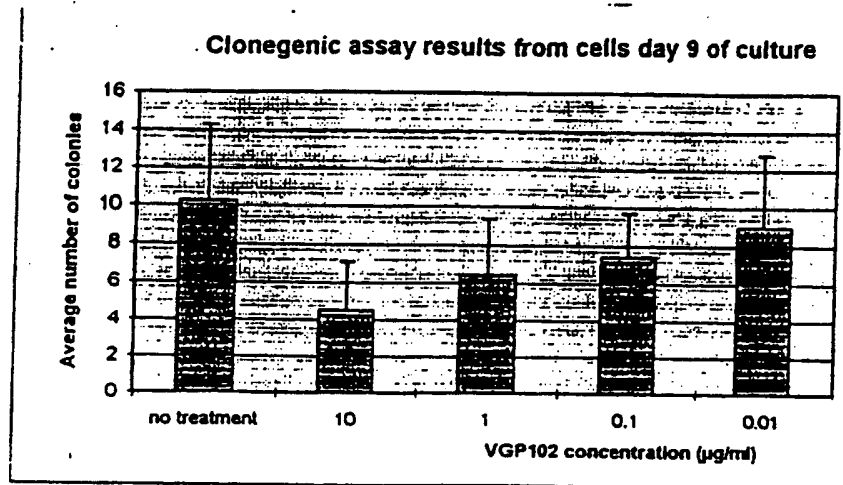
Low Mass Gate: OFF

Timed Ion Selector:

Mirror Ratio:

PSD Mirror Ratio:

Fig. 4

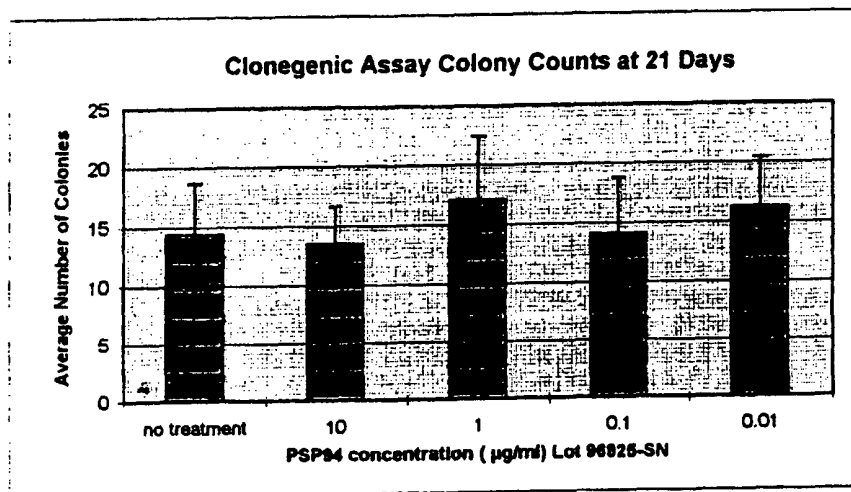
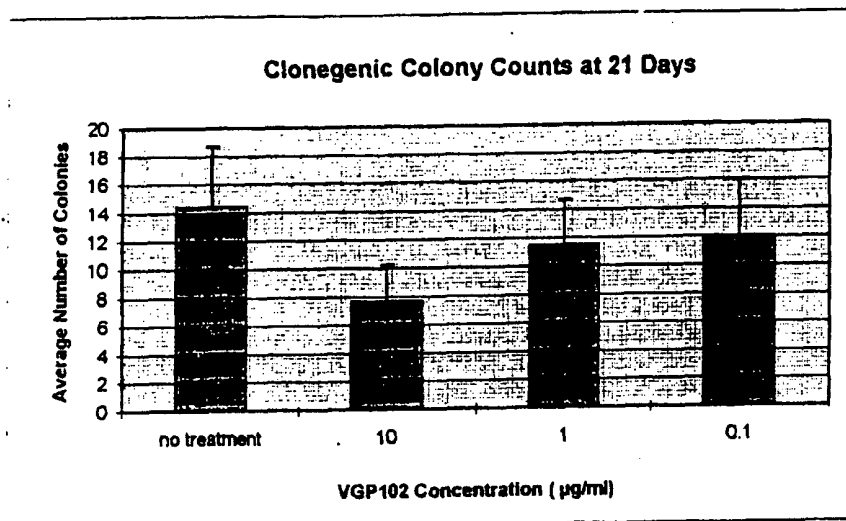


Cells seeded July 16

VGP 102 = Decapeptide (Sequence ID No. 3)

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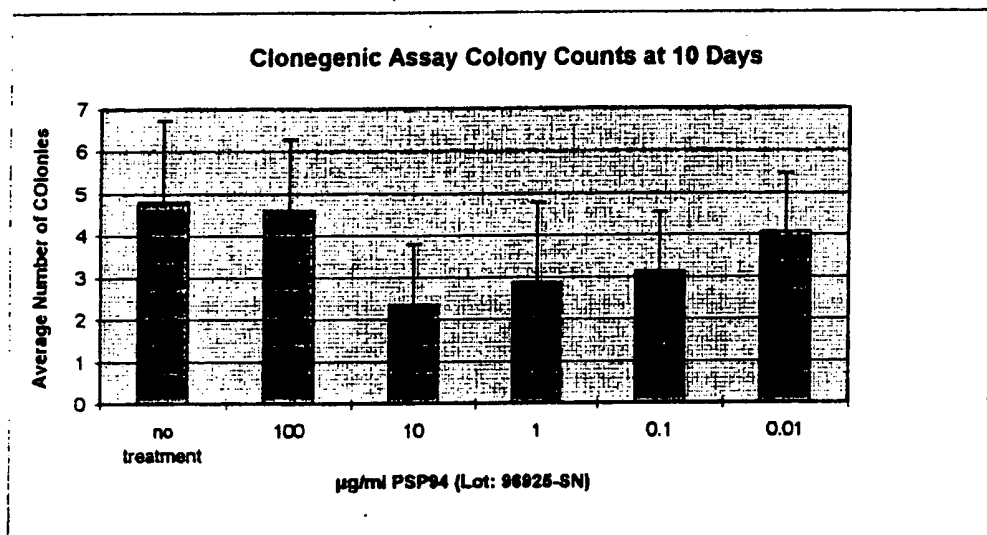
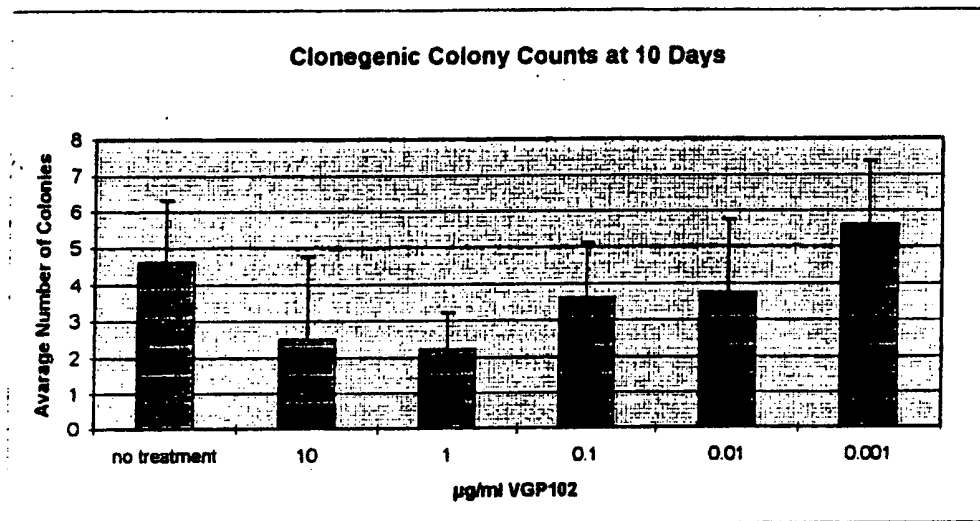
Fig. 5



cells seeded July 16.

5/17

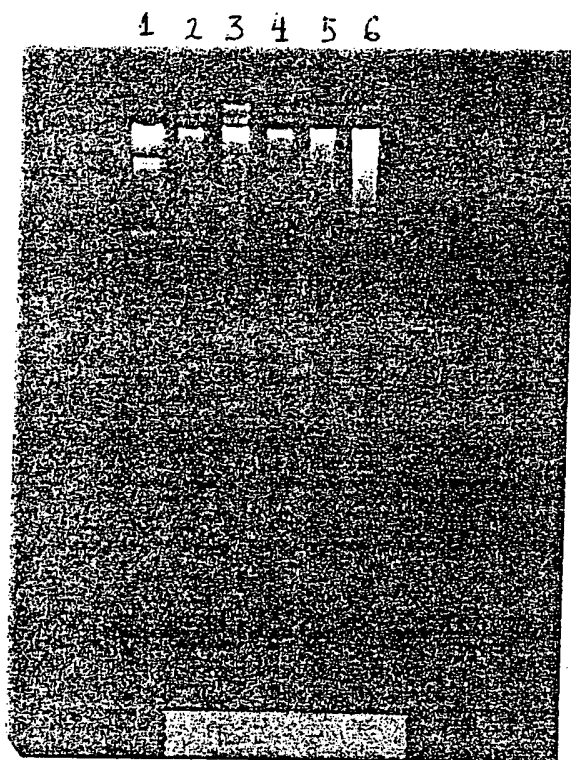
Fig. 6



Seeded July 29, 1997

Fig. 7

1. Hind III marker
2. Control
3. 40µg native PSP94
4. 20µg " "
5. 22.5µM peptide 31-45
6. 45µM peptide 31-45



DNA Fragmentation Following peptide 31-45 (Sequence ID No. 5)
Treatment of PC-3 cells.

Apoptosis assay with Elisa plus kit following peptide treatment on atcc-PC3 cells for 72hrs

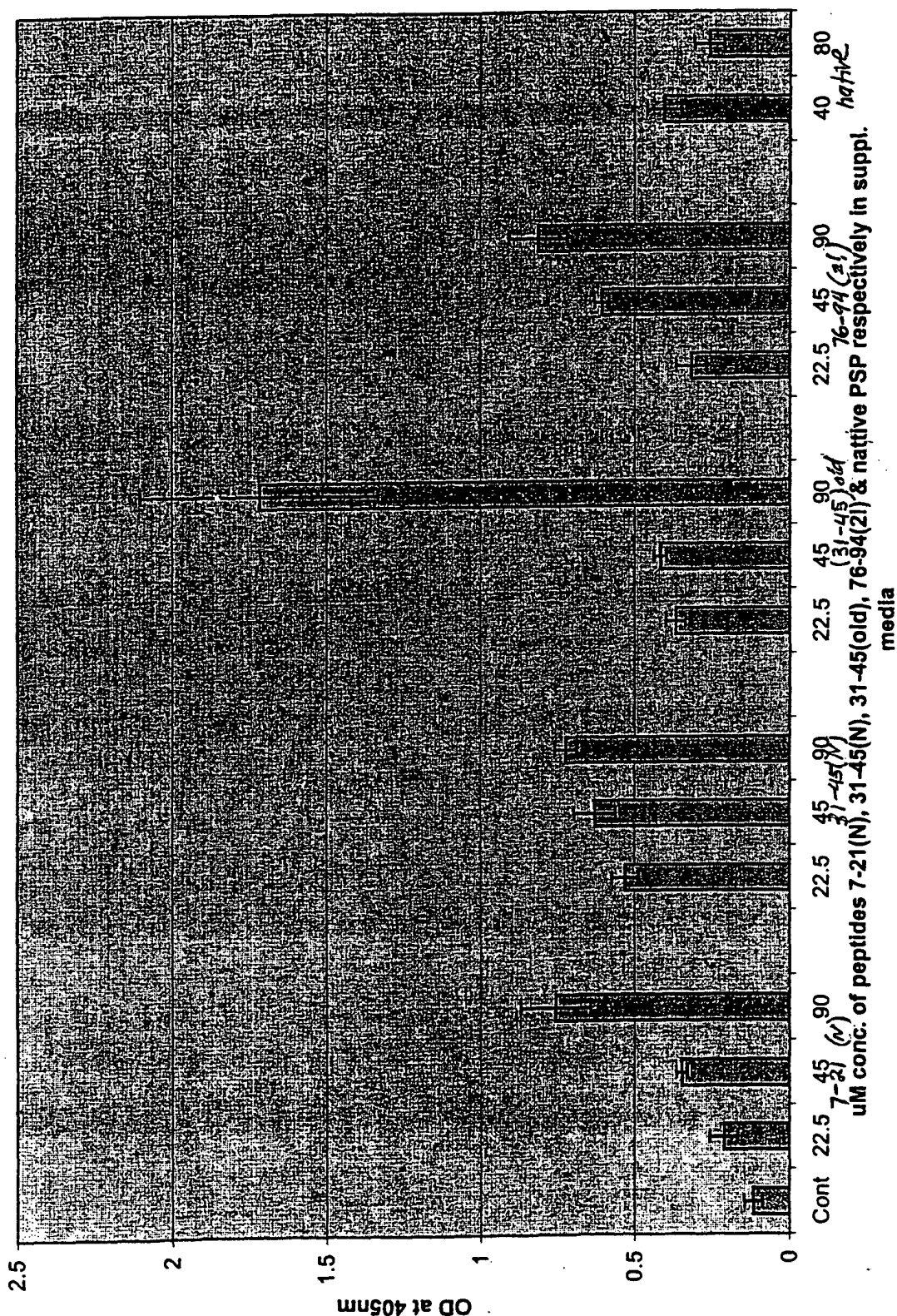
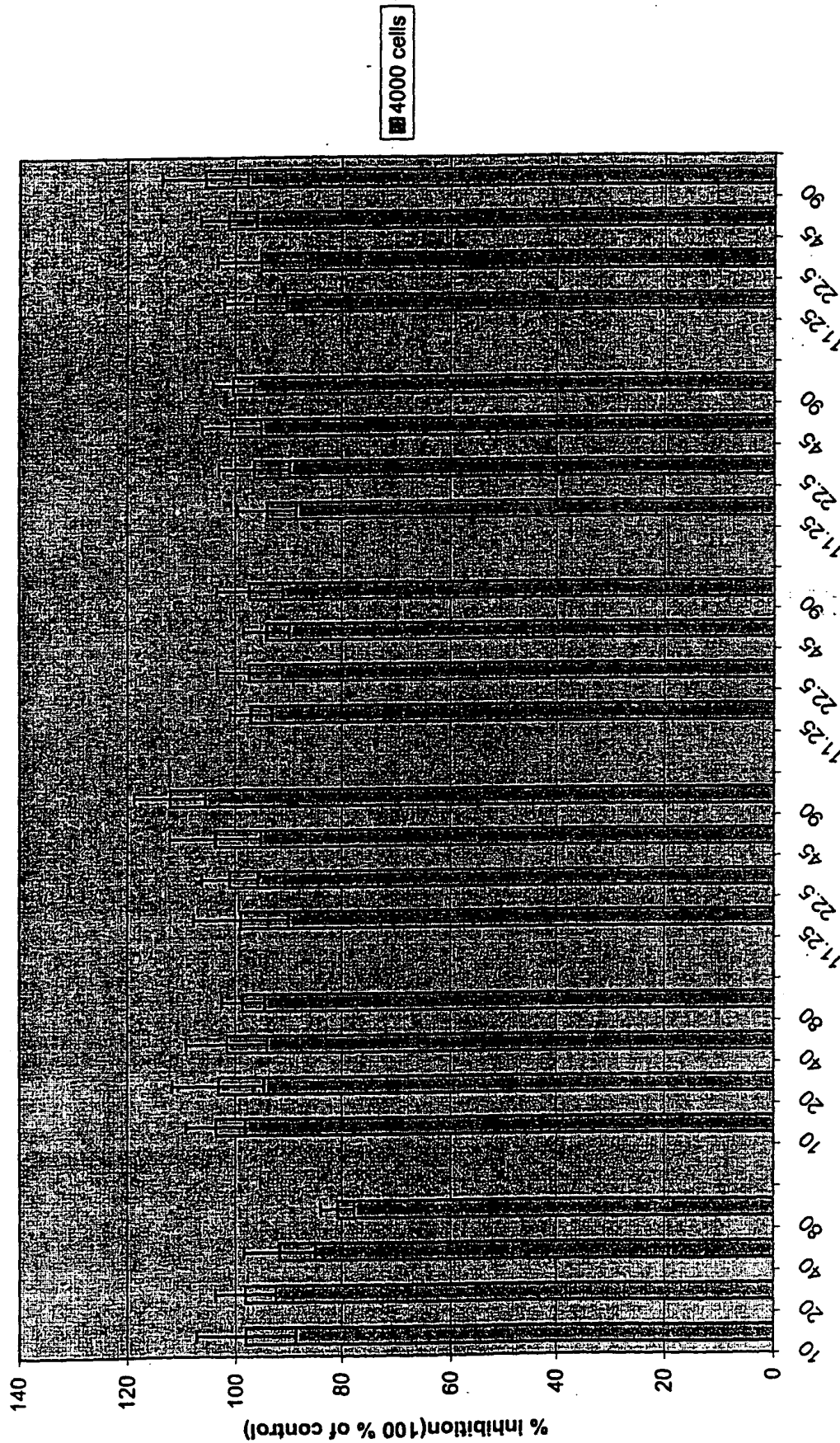


Fig. 8

Fig. 9

Growth of fibroblast cells when exposed to PSP and its analogues in vitro for 72hrs

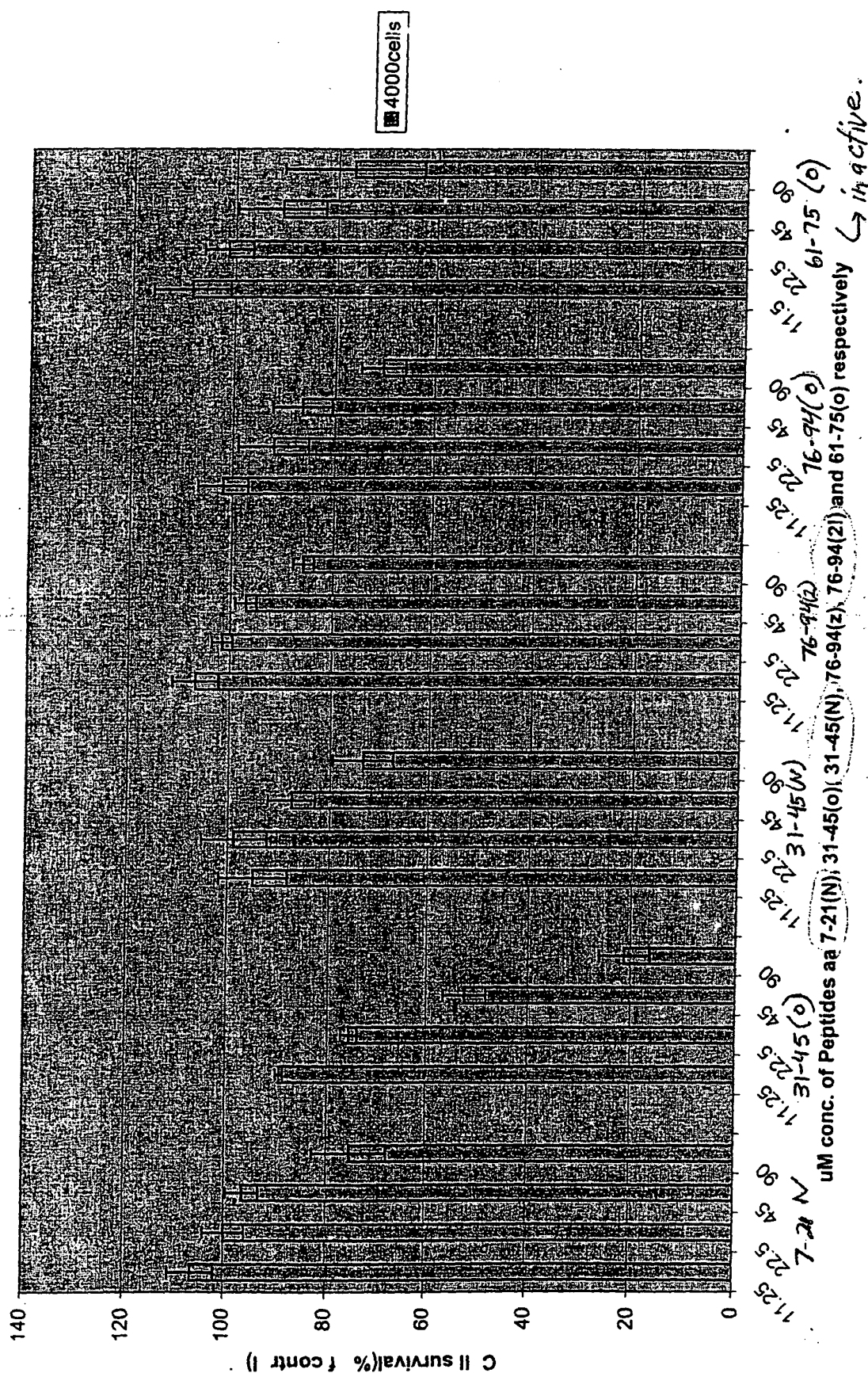


ug/ml PSP(99413-s),yPSP, uM conc of 7-21, 31-45, 76-94(2) and PB111 respectively

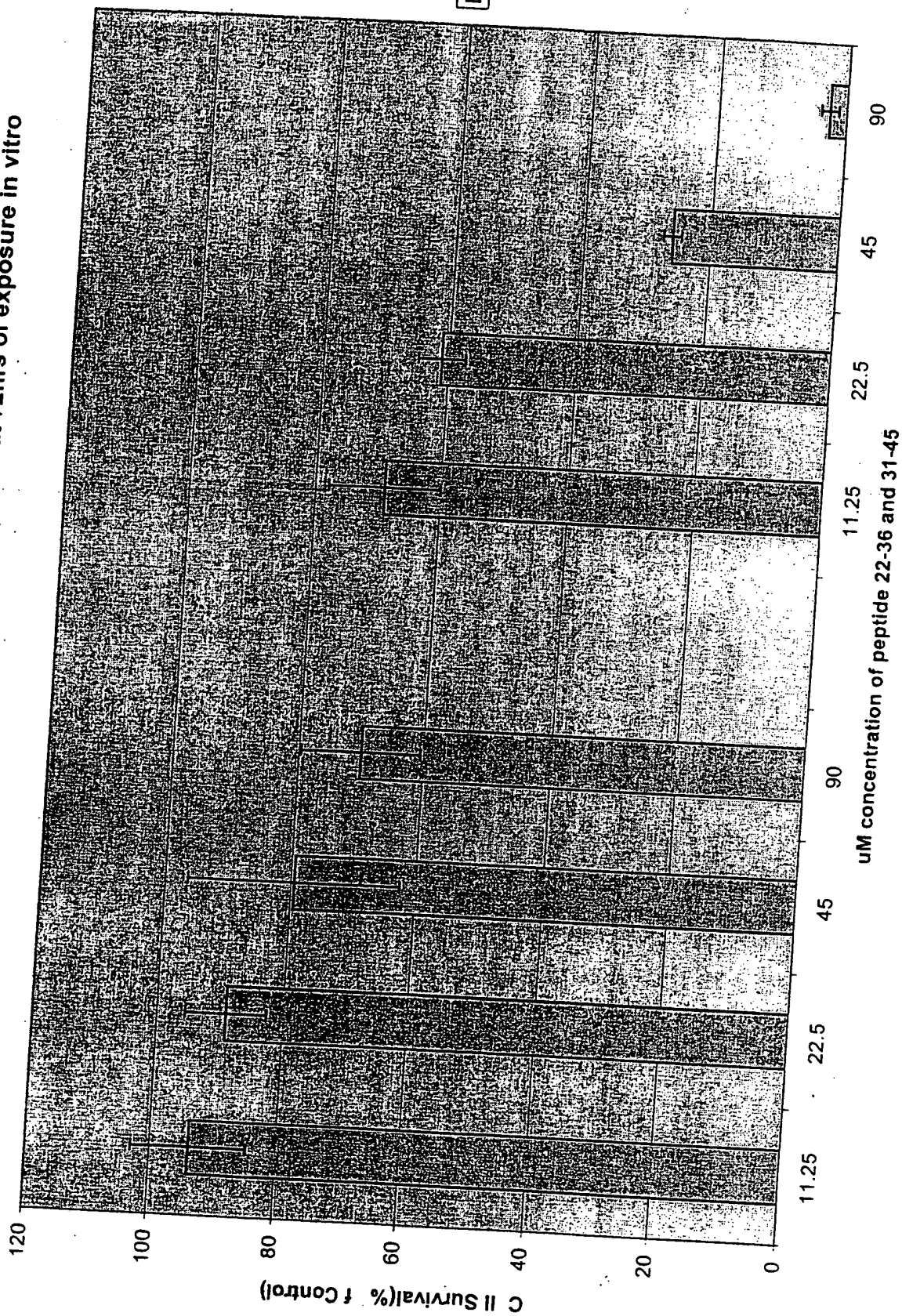
June 1, 1999

Fig. 10

Effect of Peptides on growth of ATCC-PC3 cells in vitro at 72hrs



Effect of Peptides on growth of ATCC-PC3(OPTI) cells at 72hrs of exposure in vitro



3000

Fig. 11

MLL-1 (Nude mice)

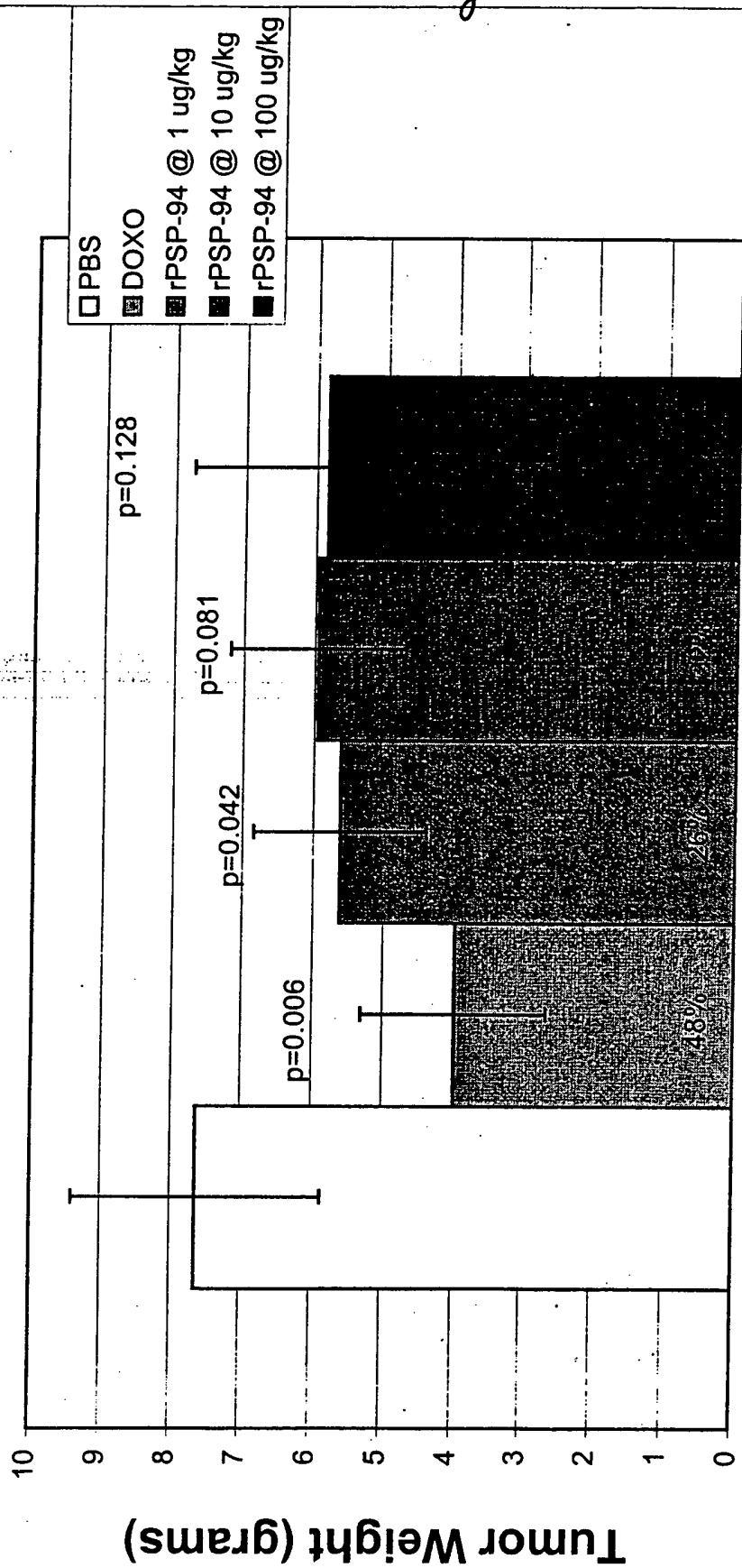
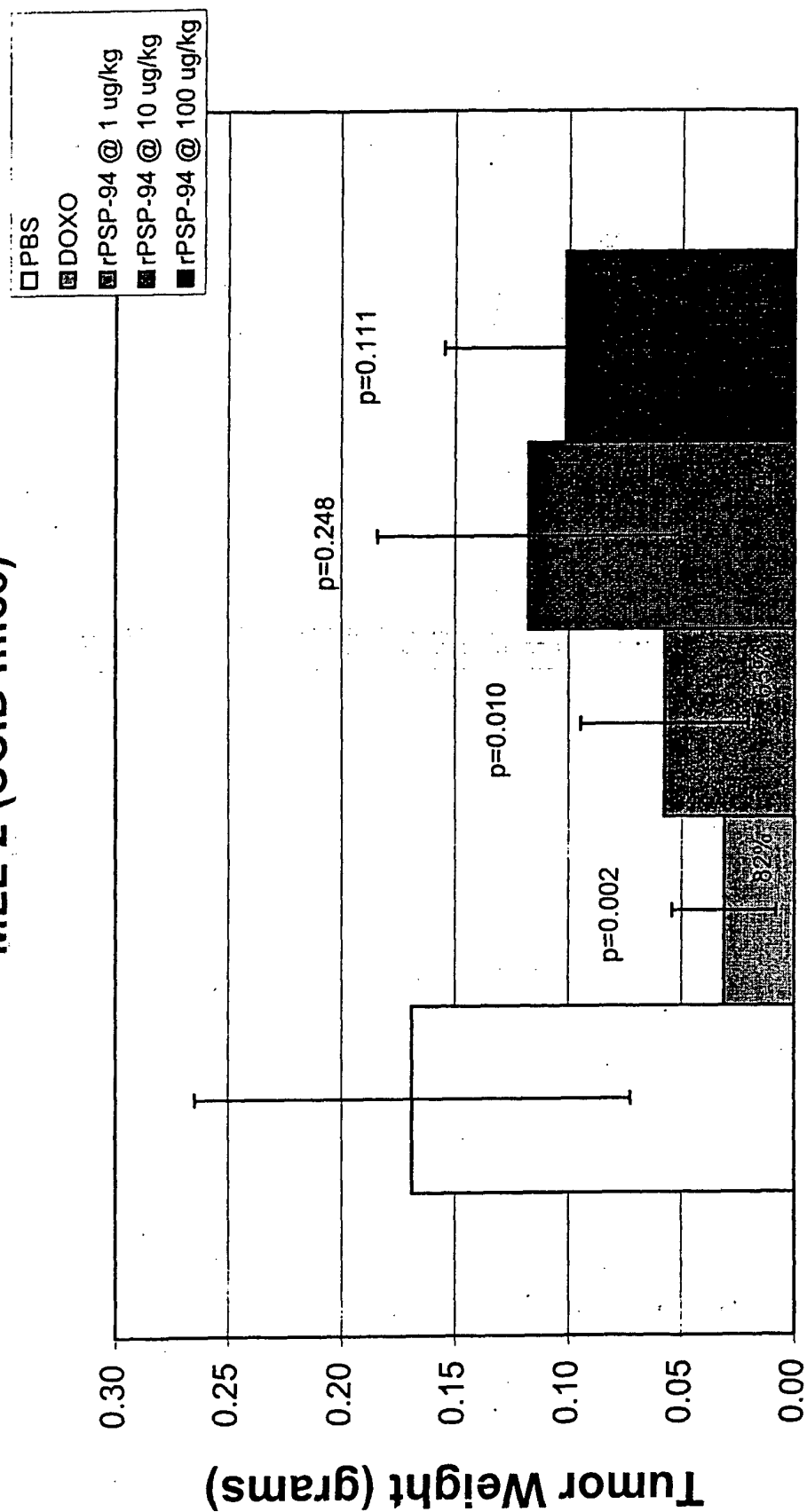


Fig. 12

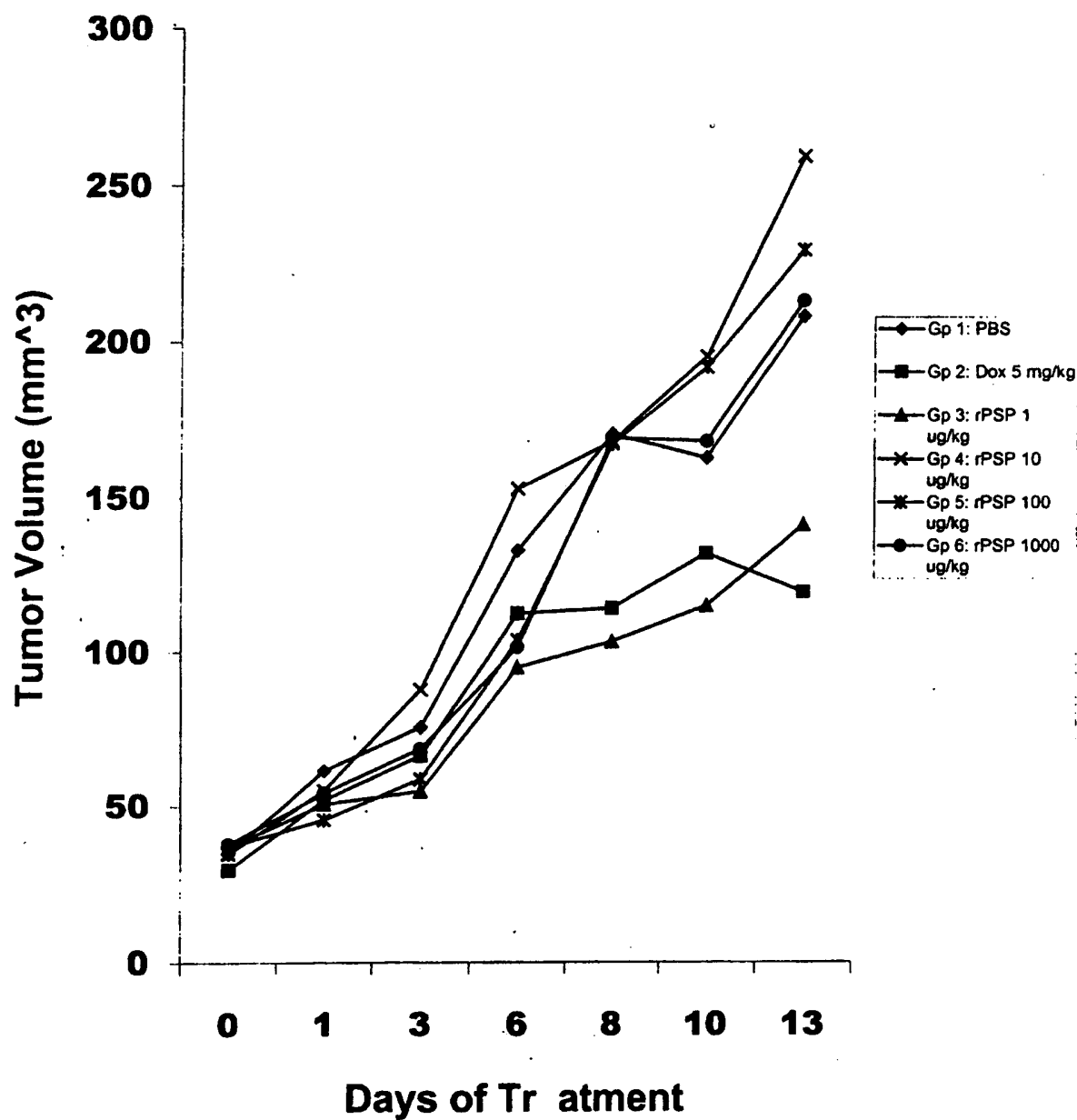
Fig. 13

MLL-2 (SCID mice)

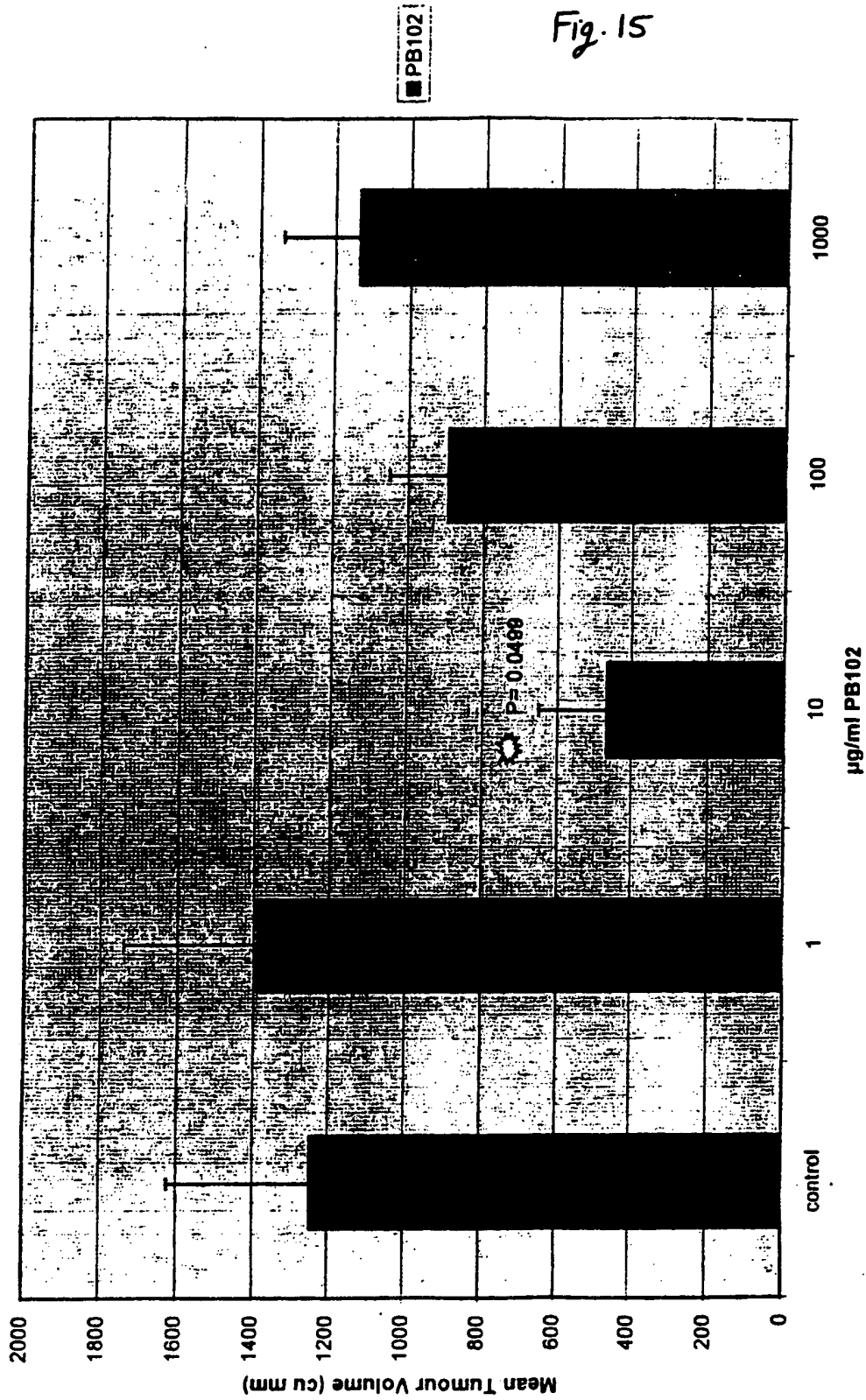


Average Tumor Weights at Day 14 p.t.i.

Fig. 14

**LAB Study 2000-2095
(Tumor Volumes)**

PB102 Dose Range Study



(Decapeptide, Sequence ID No.3)

PB111 Dose Range Study

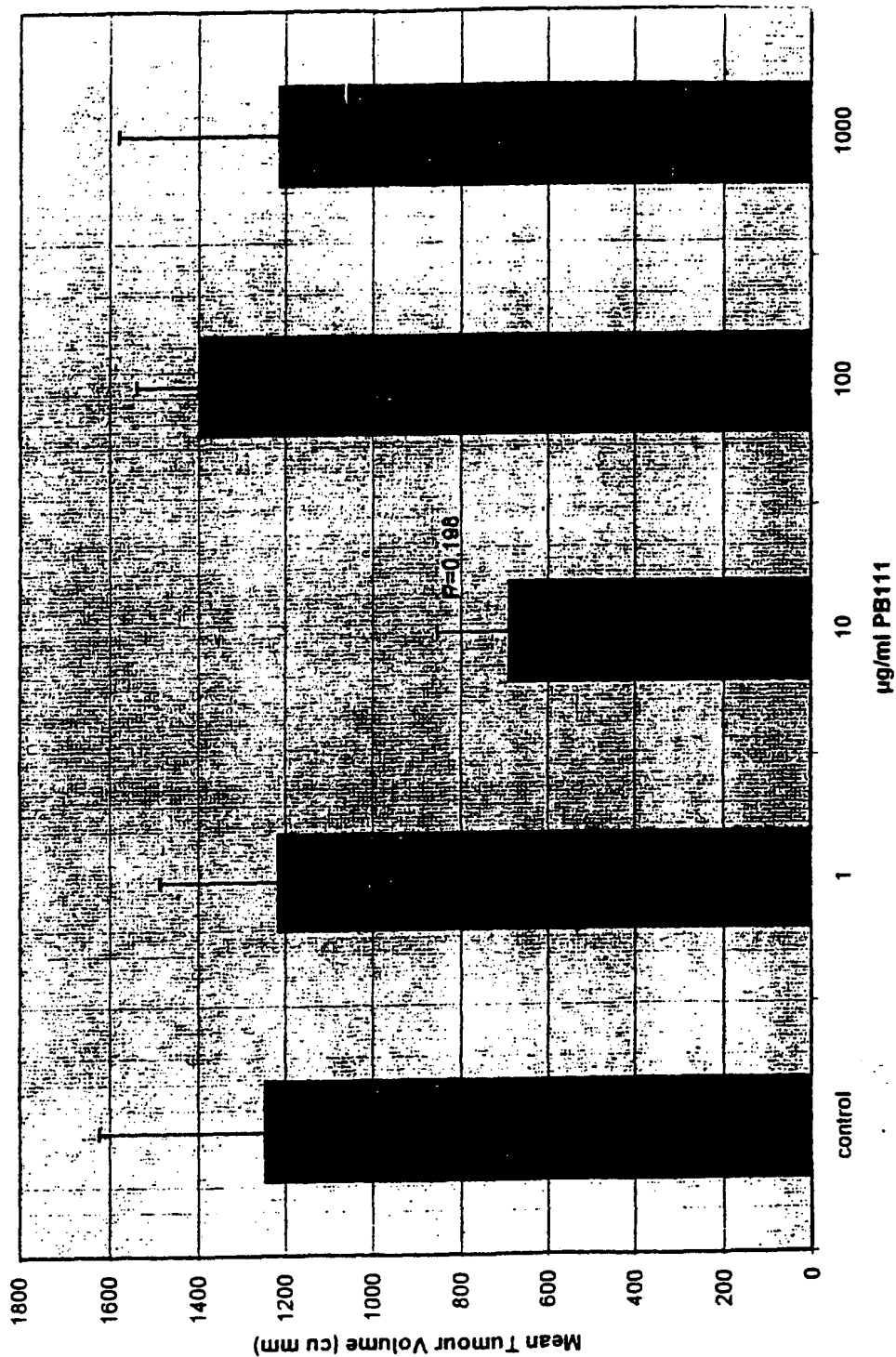


Fig. 17

PB100 Dose Range Study

